Biosurfactants production potential of native strains of *Bacillus cereus* and their antimicrobial, cytotoxic and antioxidant activities

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Abstract: Present study was designed to evaluate the biosurfactant production potential by native strains of *Bacillus cereus* as well as determine their antimicrobial and antioxidant activities. The strains isolated from garden soil were characterized as *B. cereus* MMIC 1, MMIC 2 and MMIC 3. Biosurfactants were extracted as grey white precipitates. Optimum conditions for biosurfactant production were 37°C, the 7th day of incubation, 0.5% NaCl, pH 7.0. Moreover, corn steep liquor was the best carbon source. Biuret test, Thin Layer Chromatography (TLC), agar double diffusion and Fourier Transform Infrared Spectroscopy (FTIR) characterized the biosurfactants as cationic lipopeptides. Biosurfactants exhibited significant antibacterial and antifungal activity against *S. aureus*, *E. coli, P. aeruginosa, K. pneumoniae*, *A. niger* and *C. albicans* at 30 mg/ml. Moreover, they also possessed antiviral activity against NDV at 10 mg/ml. Cytotoxicity assay in BHK-21 cell lines revealed 63% cell survival at 10 mg/ml of biosurfactants and thus considered as safe. They also showed very good antioxidant activity by ferric-reducing activity and DPPH scavenging activity at 2 mg/ml. Consequently, the study offers an insight for the exploration of new bioactive molecules from the soil. It was concluded that lipopeptide biosurfactants produced from native strains of *B. cereus* may be recommended as safe antimicrobial, emulsifier and antioxidant agent.

Keywords: Bacillus cereus, Antimicrobial, antiviral, cytotoxicity, antioxidant

INTRODUCTION

Biosurfactants are the surface active compounds commonly produced by bacteria, fungi and yeast. Lipopeptides are the well-known categories of biosurfactants mostly produced by *Bacillus* spp. consisting of cyclic peptide linked to a fatty acid chain. The major classes of lipopeptides include surfactin, iturin and fengycin (Zhang *et al*., 2016). Since the last decade, biosurfactants exposes more attractive utilization in the industry in contrast to synthetic surfactants. Biosurfactants owing to their stabilization, emulsification, antimicrobial and antioxidant properties are being gradually more preferred in diverse fields including pharmaceutical and food (Santos *et al*., 2016).

The genus *Bacillus* contains versatile species that exhibit extensive biosurfactants production (Giri *et al*., 2016). Moreover, the lipopeptides produced by *Bacillus* spp. are increasingly characterized in the recent past. However, the production for various biosurfactants has not extended up to adequate economic level. In view of the huge diversity of *Bacillus* spp. in the soil ecosystem, there is a prerequisite to isolate & preserve the indigenous strains, optimize the nutritional & environmental conditions and consume renewable substrates for biosurfactants production. In addition, determine its properties for applications in different fields (Banat *et al*., 2014). Present work aimed for the first time in the country to determine the biosurfactants production potential of native strains of *B. cereus* and evaluation of their potential use as antimicrobial, antiviral and antioxidant agent.

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MATERIALS AND METHODS

General experimental procedures

Equipment used in this study were Incubator, Hot air oven, Autoclave, Shaking incubator, Centrifuge machine, Thermal cycler, Gel documentation and Spectrophotometer whereas other materials used included inoculating loop, Micro-titration plates, Petri plates, Pipettes, test tubes, conical flask, aluminum foil etc.

Strains isolation and identification

Biosurfactant producing native strains were isolated from garden soil samples collected during January 2017, Faisalabad, Pakistan. Identification was conducted by morphological and biochemical characterization (Baindara *et al*., 2013).

Molecular characterization

The 16S rRNA gene in *Bacillus* spp. was amplified by PCR by means of the following universal primers procured from Macrogen™ Korea. Forward Primer: 27F 5'-AGAGTTTGATCMTGGCTCAG-3' Reverse primer: 1492R5'-TACGGYTACCTTGTTACGACTT-3'. Purity of amplified product was determined by 1% gel electrophoresis and size of amplicons was estimated with 100 bp ladder marker (Thermo Fisher, UK). Gene

sequencing was carried out by Macrogen Inc., Seoul, South Korea, aligned using Clustal W and maximum likelihood (ML)-based phylogenetic tree was constructed using Phylogeny.fr (Goes *et al*., 2012; Hasan *et al*., 2017).

Biosurfactants production and optimization

Inoculum was prepared in Nutrient broth (Oxoid, UK) and 20 ml was transferred to 1L of Mineral Salt Medium (MSM) [(g/L): KH_2PO_4 1.4, Na_2HPO_4 2.2, $MgSO_4$. $7H_2O$ 0.2, $CaCl_2.7H_2O$ 0.02, $FeSO_4.7H_2O$ 0.01, Yeast extract 1, NaCl 5, Glucose 10] and incubated in a rotatary shaker at 150 rpm. Each culture supernatant was subjected to drop collapse test, oil spreading technique and emulsification activity for the confirmation of biosurfactants production (Branch, 2012; Mouafi *et al*., 2016). Temperature, incubation time, salt concentration and pH for biosurfactant production were optimized. The effects of all factors were analyzed through two ways Analysis of Variance (ANOVA) at ($P<0.05$) using Minitab® Version 16 (Abdel-Mawgoud *et al*., 2008).

Characterization biosurfactants

Biuret test was carried out for the presence of amino acids in biosurfactants (Nitschke and Pastore, 2006). Agar double diffusion technique was carried out to determine the ionic characters. Cetyl Trimethyl Ammonium Bromide (CTAB) was used as cationic whereas Sodium Dodecyl Sulphate (SDS) anionic substance. Presence of precipitation line between the wells revealed the ionic character of biosurfactants (Rufino *et al*., 2014). Biosurfactant sample was dissolved in chloroform and spotted on silica gel 60 F_{254} plate (Merck, USA) and thin layer chromatography (TLC) was performed as described by Cao *et al*. (2009). Functional groups and overall nature of chemical bonds in biosurfactant samples were analyzed by Fourier Transform Infrared Spectroscopy (FTIR) and spectral data were collected over the range of 450-4000 cm^{-1} (Joshi *et al*., 2016).

Antibacterial and antifungal activity

Antibacterial activity was determined against *Staphylococcus aureus*, *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae* and antifungal activity against *Aspergillus niger* and *Candida albicans* using standard agar well diffusion and Micro-broth dilution methods. The diameters of zones of inhibition were measured in mm and results were interpreted as sensitive, intermediate and resistant. The lowest concentration of biosurfactant inhibited the growth was considered as Minimum Inhibitory Concentration (MIC) (Fernandes *et al*., 2007).

Antiviral activity

In-vitro antiviral activity of biosurfactants against New Castle Disease Virus (NDV) was determined by the haemagglutination test (HA) (Huang *et al*., 2006). The reduction in Haemagglutination titer of NDV was observed and calculated by the following formula.

$$\text{Reduction in HA titer} = \frac{\text{HA titer of virus} - \text{HA titer of virus treated with biosurfactant}}{\text{HA titer of virus}} \times 100$$

Cytotoxicity testing

Cytotoxicity of biosurfactants was determined by MTT colorimetric (3-(4, 5- dimethylthiazol-2-yl) - 2, 5-diphenyl tetrazolium bromide) assay. Two-fold serial dilutions of biosurfactants (10mg/ml) were prepared in deionized water. The cell survival was determined in Baby Hamster Kidney (BHK-21) cell lines for each dilution and percentage cell survival was calculated (Cao *et al*., 2009).

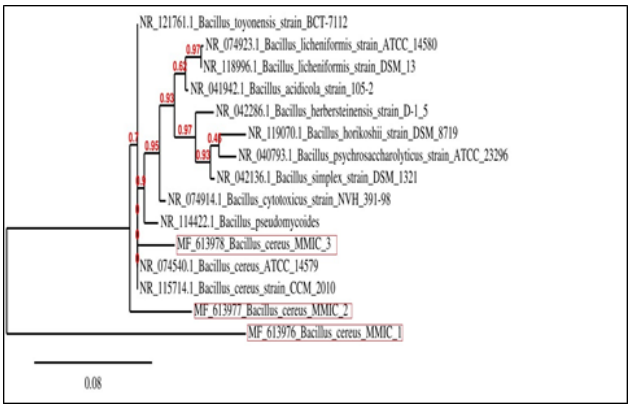

Fig. 1: Phylogenetic tree constructed on the basis of Maximum Likelihood of 16s RNA gene

Antioxidant activity

Antioxidant activity was evaluated by Ferric-reducing activity and 1, 1- diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity. Reducing power of biosurfactants was compared with Butylated Hydroxytoluene (BHT). The absorbance at 700 nm was measured spectrophotometrically (Thermo Scientific, UK). Increased absorbance was designated as increased reducing power (Jemil *et al*., 2017). The antioxidant potential of biosurfactants was evaluated on the basis of their scavenging activity of DPPH free radical as described by Kadaikunnan *et al*. (2015).

RESULTS

Three biosurfactant producing bacterial strains were characterized as *B. cereus* MMIC1, MMIC 2 & MMIC 3 and submitted to the Genbank under the Accession No. MF613976, MF613977 & MF613976, respectively (Fig. 1). The result of drop collapsing method, oil spreading technique and emulsification activity indicated positive results by all three strains. The maximum amount of biosurfactant obtained at 37°C was 4.4 g/L, 3.3 g/L & 2.3 g/L by *B. cereus* strains MMIC 1, MMIC 2 and MMIC 3, respectively. Likewise, optimum yield obtained from MMIC 1, MMIC 2 and MMIC 3 at 7th day of incubation

Table 1: Antibacterial and antifungal activity of biosurfactants by disc diffusion method and micro-broth dilution method

Bacterial and fungal isolates	Zones of inhibition (Mean ± Standard deviation) mm			MIC (mg/ml)
	Concentration of biosurfactant (mg/ml)			
	10	15	30	
S. aureus	16.1±0.5	17.2±0.5	20.2±0.3	0.5±0.76
E. coli	13.9±0.5	15±0.5	20.2±0.3	1.04±1.47
P. aeruginosa	13.6±0.4	14.1±0.2	16±0.3	2.08±1.2
K. pneumoniae	13.1±0.4	14.5±0.3	15±0.2	4.16±3.41
C. albicans	10.2±0.2	11.2±0.3	12.8±0.3	7.6±3.2
A. flavus	10.2±0.2	11.4±0.3	11.4±0.3	7.6±3.2

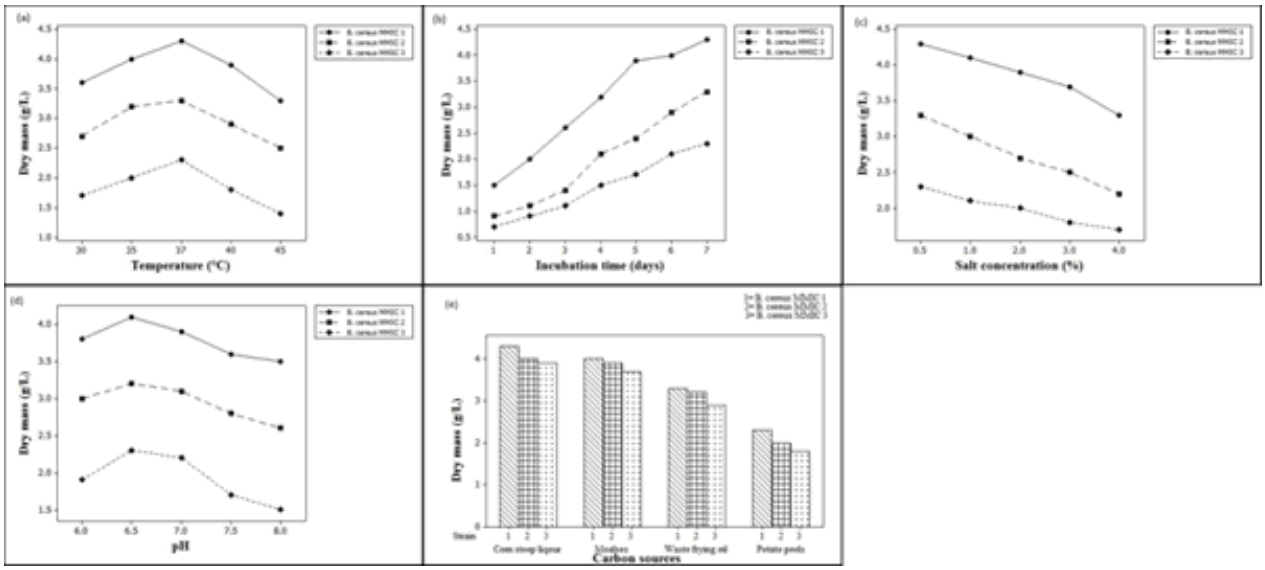

Fig. 2: Optimization of conditions for biosurfactant production by strains of *B. cereus* (a) Temperature (b) Incubation time (c) Salt concentration (d) pH (e) Carbon sources

was 4.3g/L, 3.3g/L and 2.3g/L, respectively. Biosurfactants produced by MMIC 1, MMIC 2 and MMIC 3 at 0.5% NaCl was 4.3g/L, 3.3g/L and 2.3g/L, respectively. Similarly, maximum amount recovered by MMIC 1, MMIC 2 and MMIC 3 at pH 6.5 was 4.2g/L, 3.2g/L & 2.2g/L, respectively. The maximum biosurfactant production with achieved with corn steep liquor (4.3g/L, 4.0g/L & 2.5g/L) by all three strains (fig. 2).

Biuret test indicated the presence of peptides whereas agar double diffusion test revealed the cationic nature of biosurfactants. The two spots were visualized in TLC when silica gel plate was sprayed with iodine. In FTIR spectra, characteristics peak between 3000-3300 cm^{-1} indicated the N-H bond. The peak at 2100-2150 indicated week intensity of C≡C. The bond at 1690-1720 corresponds to the presence of C=O. Moreover, the bond at 1550-1640 suggested the presence of stronger amides. The bonds at 1300-1420 and 1020-1180 represented $–CH_3$ and C-H bonding, respectively. The peaks at 650-680 demonstrated the secondary amide structure (fig. 3).

In agar well diffusion method, the highest zones of inhibition was found against *S. aureus* (20.17±0.31) mm and lowest against *A. flavus* (12.7±0.9) mm at 30 mg/ml. In micro-broth dilution method, the lowest MIC was recorded against *S. aureus* (0.52±0.76) mg/ml and highest against *A. flavus* (7.6±3.2) mg/ml (table 1). However, *In-vitro* antiviral activity against NDV indicated that as the concentration of biosurfactant increased, the percentage of titer reduction was also increased. Moreover, maximum titer reduction (87%) was reported at a concentration of 10mg/ml. The cell survival percentage increased from 63% to 92% as the concentration decreased from 10mg/ml to 5mg/ml by MTT assay of BHK-21 cell lines. The ferric-reducing activity indicated that the highest absorbance was recorded at 2 mg/ml. The DPPH scavenging activity was found in the range of 27% to 63% with a concentration of 0.5 to 2.0 mg/ml (fig. 4).

DISCUSSION

In the current study, biosurfactant production potential of *B. cereus* isolated from garden soil was evaluated. Soil is recognized as a rich source and habitat of *Bacillus* spp.

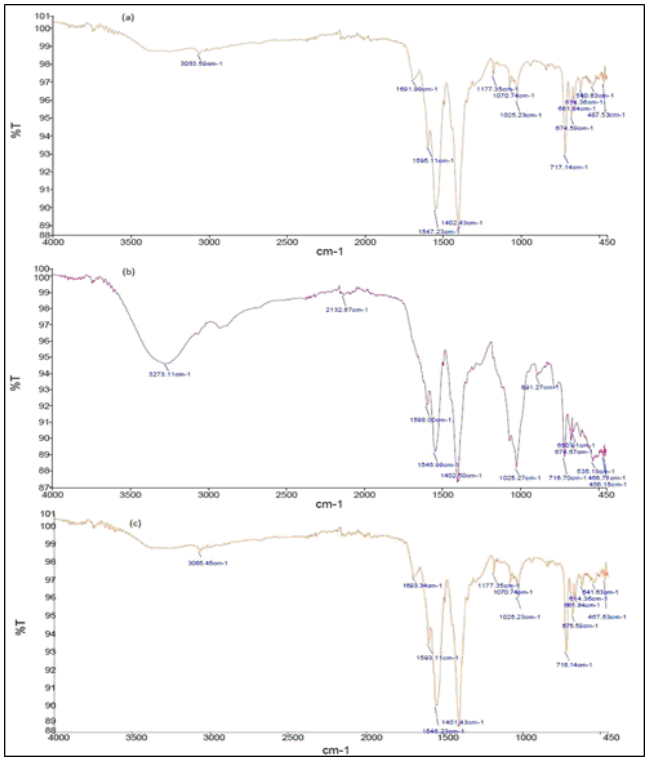

Fig. 3: Infrared spectrum of biosurfactants produced by strain of (a) *B. cereus* MMIC 1 (b) *B. cereus* MMIC 2 (c) *B. cereus* MMIC 3

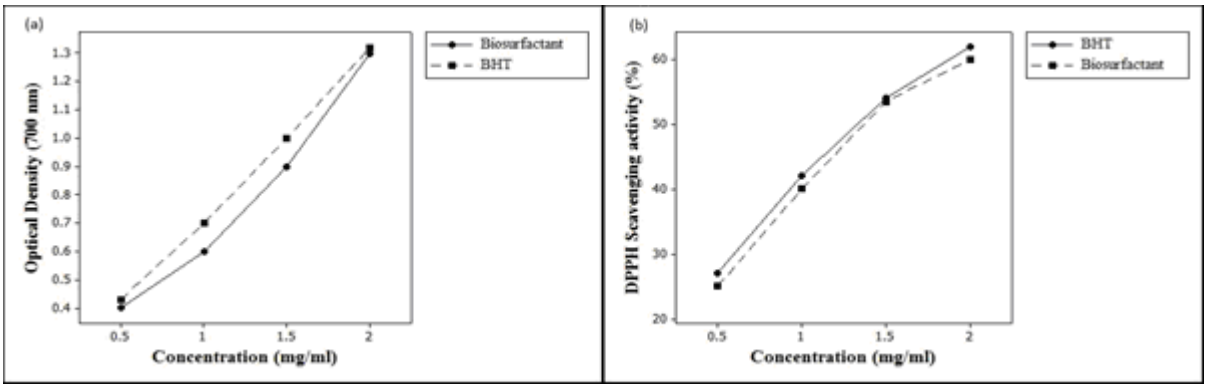

Fig. 4: Antioxidant activity of biosurfactants by (a) Ferric-reducing activity (b) DPPH scavenging activity (%)

which are capable of forming biosurfactants. Many scientists reported the biosurfactant production by *B. subtilis, B. licheniformis, B. pumilus, B. amyloliquefaciens, B. salmalaya, B. atrophaeus, B. brevies* and *B. mojavensis* (Zhang *et al*., 2016; Mouafi *et al*., 2016; Joshi *et al*., 2016). However, the several studies supported the biosurfactant production by *B. cereus* (Sriram *et al*., 2011).

Two-way ANOVA demonstrated that means were significantly different ($P<0.05$) at different temperatures, incubation time, pH, salt concentration and carbon sources by three strains of *B. cereus*. The optimum conditions for biosurfactant production was 37°C, 7th day of incubation, 0.5%NaCl, pH and corn steep liquor was served as best carbon source. According to Mouafi *et al*., (2016), *B. brevis* produced the biosurfactants on the 10th day of incubation period. However, Gomaa (2013) reported the biosurfactant production on the 7th day of incubation by *B. licheniformis* strain M104. Biosurfactant production was inhibited up to 10% NaCl concentration. The optimum pH for biosurfactant production by *B. subtilis* was 6.8-6.5 (Abdel-Mawgoud *et al*., 2008). Likewise, other scientist reported the biosurfactant production with corn steep liquor and regarded as the best carbon source. Their productivity was reached at 1.12g with molasses (Abdel-Mawgoud *et al*., 2008).

The presence of peptide bond matched with results was reported by Yadav *et al*., (2016). Agar double diffusion test indicated that extracted biosurfactant was cationic. In contrast to this, biosurfactants produced by *Candida*

lipolytica formed precipitation line with a cationic surfactant and regarded as anionic biosurfactant (Rufino *et al*., 2014). The spots visualized in the TLC indicated the presence of lipopeptides. The FTIR spectrum showed the presence of amino and hydrocarbon groups which suggests the production of a lipopeptide biosurfactant (Zhang *et al*., 2016).

One of the potential uses of lipopeptide biosurfactant as bio-product includes its role as antimicrobial agent. Lipopeptide biosurfactants have revealed their antimicrobial activity by their lytic membrane properties. The results were in accordance with previously reported antibacterial, antifungal and antiviral activity of biosurfactants (Gomaa, 2013; Jemil *et al*., 2017). The concentration having cell survival percentage ≥50% was taken as non-toxic or safe. Thus, biosurfactants produced by native strains of *B. cereus* had 63% cell survival up to 10 mg/ml concentration and were declared as safe or non-toxic. Thus they may be used as potential antimicrobial agents in humans and animals (Cao *et al*., 2009). According to results, biosurfactant presented the capacity to donate hydrogen, therefore display DPPH scavenging activity. Additionally, the reducing power of biosurfactants was improved in a dose-dependent response indicated that some functional groups present in biosurfactants were both electron recipients and electron donors to convert them into more stable compounds. The antioxidant activity of biosurfactants when compared with BHT indicated that both have similar results (Jemil *et al*., 2017).

CONCLUSION

It was concluded that all three indigenous strains (MMIC 1, MMIC 2 and MMIC 3) of *B. cereus* had significant biosurfactants producing potential. Biosurfactants belonged to lipopeptides class and were stable at a wide range of temperature and pH. Corn steep liquor was found to be the best carbon source for optimum yield. Biosurfactants possessed very good antibacterial, antifungal and antiviral activity. They also exhibited excellent antioxidant properties by ferric reducing activity and DPPH scavenging activity. It is anticipated that in future, super-active biosurfactants will be produced at industrial level using agro-industrial wastes and will be used in food and pharmaceutical industries at commercial scale.

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